Senescence of the adaptive immune system in health and aging-associated autoimmune disease

van der Geest, Kornelis Stephan Mario

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AGING-DEPENDENT DECLINE OF IL-10 PRODUCING B CELLS COINCIDES WITH PRODUCTION OF ANTINUCLEAR ANTIBODIES BUT NOT RHEUMATOID FACTORS

Kornelis S.M. van der Geest[1†], Pedro G. Lorencetti[1†], Wayel H. Abdulahad[1], Gerda Horst[1], Minke Huitema[1], Caroline Roozendaal[2], Bart-Jan Kroesen[2], Elisabeth Brouwer[1], Annemieke M.H. Boots[1]

[1] Department of Rheumatology and Clinical Immunology, [2]Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands.

† Contributed equally to the study

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Summary

Aging is associated with development of autoimmunity. Loss of B cell tolerance in the elderly is suggested by an increased prevalence of anti-nuclear antibodies (ANAs) and rheumatoid factors (RFs). Accumulating evidence indicates that B cells also impact autoimmunity via secretion of cytokines. So far, few studies have directly assessed the effect of aging on the latter B cell function. Here, we determined if and how human aging influences the production of cytokines by B cells. In a cross-sectional study, we found that absolute numbers of circulating B cells were similar in 31 young (age 19-39) and 73 old (age ≥60) individuals. Numbers of transitional B cells ($CD19^{+}CD27^{-}CD38^{High}CD24^{High}$) were decreased in old individuals, whereas numbers of naive and memory B cell subsets were comparable in young and old individuals. Short-term in vitro stimulation of whole blood samples revealed that numbers of B cells capable of producing TNF-α were similar in young and old individuals. In contrast, B cells capable of IL-10 production were decreased in old subjects. This decline of IL-10^{+} B cells was observed in old individuals that were ANA positive, and in those that were negative for both ANAs and RFs. However, IL-10^{+} B cells were remarkably well retained in the circulation of old subjects that were RF positive. Thus, pro-inflammatory TNF-α^{+} B cells are retained in the elderly, whereas IL-10^{+} B cells generally decline. In addition, our finding findings indicate that IL-10^{+} B cells may differentially impact the development of ANAs and RFs in the elderly.

Introduction

Aging is associated with development of autoimmunity (Boots et al, 2013). The incidence of various autoimmune diseases, such as rheumatoid arthritis, increases substantially with age (Crowson et al, 2011). Moreover, the prevalence of autoantibodies is increased in serum samples of healthy, elderly subjects (Nisihara et al, 2013; van Schaardenburg et al, 1993). These findings suggest that B cell tolerance may be compromised in the elderly.

B cells play an important role in systemic autoimmune diseases via production of autoantibodies. Prototype autoantibodies are rheumatoid factors and anti-nuclear antibodies. The seroprevalence of rheumatoid factors and anti-nuclear antibodies increases with age (Nisihara et al, 2013; van Schaardenburg et al, 1993). In recent years, it has become clear that B cells also promote autoimmunity via secretion of pro-inflammatory cytokines, such TNF-α and IL-6 (Barr et al, 2012; van der Geest et al, 2014a). So far, little is known about the impact of age on the frequencies of these pro-inflammatory B cells. In addition, various authors have described B cells that secrete anti-inflammatory cytokines, such as IL-10 and TGF-β (Blair et al, 2010; Iwata et al, 2011). Recently, Duggal et al. have convincingly demonstrated that aging compromises the in vitro potential of transitional B cells to differentiate into IL-10 producing B cells (Duggal et al, 2013). Furthermore, this poor differentiation potential was associated with rising titres of rheumatoid factors in elderly subjects. It remains to be elucidated, however, if aging also influences the actual number of IL-10 producing B cells in the circulation of aged individuals, and whether such modulation is indeed associated with increased prevalence of rheumatoid factors, and perhaps anti-nuclear antibodies.

In the current study, we first investigated the impact of healthy aging on the proportions of circulating TNF-α and IL-10 producing B cells. Subsequently, we analysed serum samples of healthy young and old subjects for presence of rheumatoid factors and anti-nuclear antibodies. Finally, we tested whether the presence of these autoantibodies was associated with differential maintenance of TNF-α and IL-10 producing B cells in aged individuals.

Materials and Methods

Study subjects and samples

Peripheral blood was collected from healthy, young (n=31) and old (n=73) individuals. Seven young and 26 old individuals were male. Representative characteristics and details on recruitment and health assessment of study participants were described previously (van der Geest et al, 2014b). Written informed consent was obtained from all study participants and the study was approved by the Medical Ethical Committee of the UMCG. All procedures were in accordance with the Declaration of Helsinki.

Quantification of B cells and B cell differentiation subsets

B cells and B cell differentiation subsets were enumerated as reported previously (van der Geest et al, 2014a). In brief, absolute numbers of CD19+ B cells were determined according to the MultiTest TruCount method (BD). Mononuclear cells were isolated from heparinized blood with Lymphoprep (Axis-Shield) and stained with the following monoclonal antibodies: CD19-efluor605, CD24-PcP-efluor710, CD27-APC-efluor780, CD38-PE-Cy7 (all eBioscience), IgD-V450 and IgM-APC (BD). Samples were measured on a LSR-II (BD) flow cytometer and analysed with Kaluza Flow Analysis Software (Beckman Coulter).

Intracellular cytokine staining

Heparinised blood samples were diluted 1:1 with RPMI and stimulated with 40 nM PMA and 2 nM Ca^{2+} ionophore A23187 in the presence of 3 μM brefeldin A (BFA, Sigma) during 4 hours. After red blood cell lysis with ammonium chloride, stimulated cells were treated with Fix/Perm reagent A and B (Invitrogen) and stained with the following antibodies: CD19-APC-efluor780 (eBioscience), CD22-FITC, IL-10-APC (BD) and TNF-α-PcP-Cy5.5 (Biolegend).

Detection of anti-nuclear antibodies and rheumatoid factors

To measure anti-nuclear antibodies, dilutions of sera (1:40 and 1:80) were incubated on slides coated with human epithelioma type 2 cells transfected with SSA/Ro 60 kD antigen (Hep2000 cells, BioMedical Diagnostics). Next, slides were incubated with goat-anti-human IgG-FITC (BioMedical Diagnostics). Subsequently, slides were analysed for the presence of ANAs with a Leica DM LB2 fluorescence microscope (Leica Microsystems) by an experienced technician.

Serum levels of IgM rheumatoid factor were determined by ELISA. Costar 96-well plates were coated with aggregated human IgG. Following coating, dilutions of serum samples were incubated. Next, goat anti-human IgM-HRPO (Southern Biotech) was added. Finally, wells were incubated with TMB and the reaction was stopped with H_2SO_4. Plates were scanned on a Versamax reader (Molecular Devices). Sera of rheumatoid factor positive individuals with known concentrations of IgM rheumatoid factors were used to quantify levels of IgM rheumatoid factors in the tested samples. An ANA titre ≥ 40 and an IgM RF level of >15 IU/mL were considered positive.

Statistics

Young and old subjects were compared with the Mann-Whitney U test. P values less than 0.05 (2 tailed) were considered statistically significant. Analyses were performed with IBM SPSS Statistics 20 (SPSS) and GraphPad Prism 5.0 software.

Results

Limited effect of aging on B cell numbers

To study whether B cells are modulated with age, we first investigated the absolute number of $CD19^+$ B cells in the peripheral blood of healthy, young (age 19-39) and old (age ≥ 60) individuals. Absolute numbers of circulating B cells were comparable in young and old individuals (Figure 1A, left panel). In addition, B cell numbers remained stable in longitudinal study of old study subjects (Figure 1A,

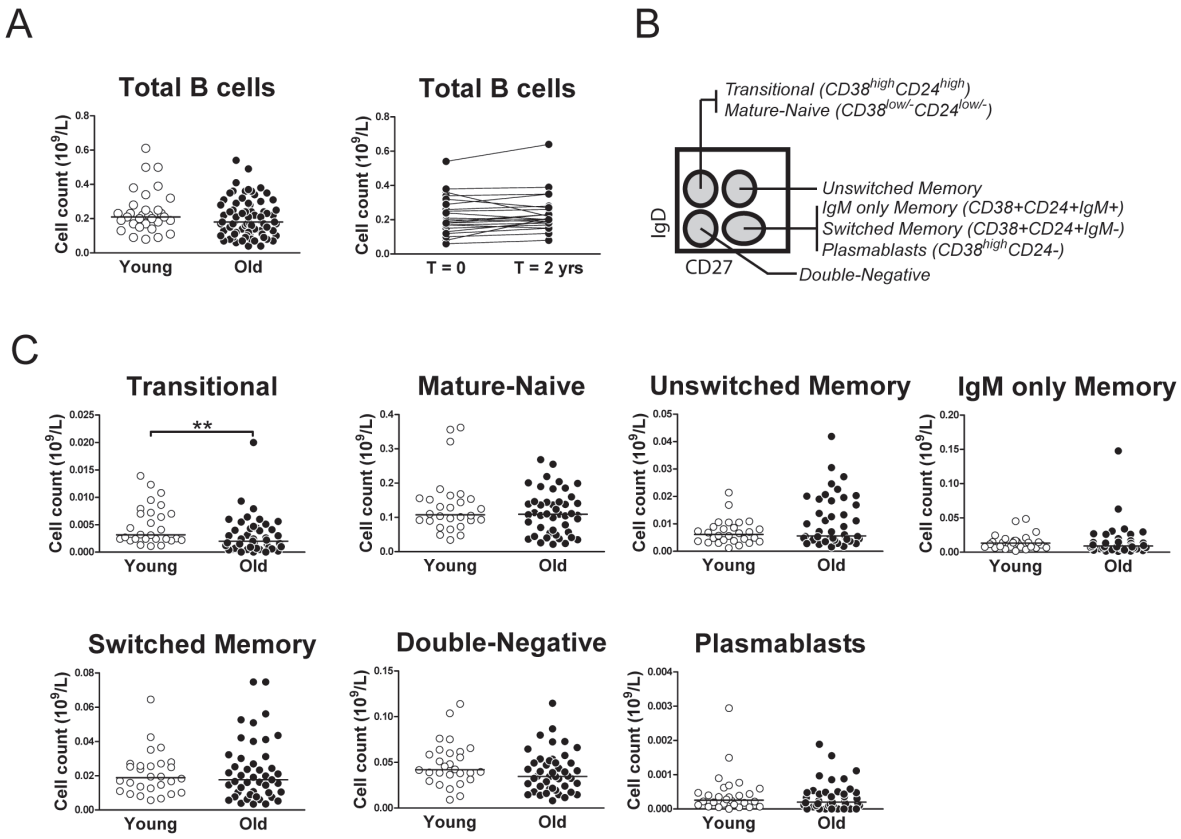

Figure 1. Effect of age on circulating B cell numbers and B cell differentiation subsets. (A) Absolute numbers of CD19+ B cells in peripheral blood of 31 young and 73 old individuals (left panel). In addition, absolute B cell numbers were determined in 22 old individuals after 2 years of follow up (right panel). (B) Flow cytometric gating strategy for distinct B cell differentiation subsets (left panel) and representative CD27/IgD staining in peripheral blood CD19+ B cells from a young and an old healthy individual (right panel). (C) Absolute numbers of distinct B cell differentiation subsets in 28 young and 45 old individuals. Statistical significance by Mann Whitney U test is indicated as ** $p < 0.01$.

right panel). Furthermore, no differences were observed between B cell counts in male and female donors (Supplemental Figure 1).

To determine whether particular B cell differentiation subsets were affected by aging, we further delineated B cells according to their expression of multiple differentiation markers (Figure 1B; Supplemental Figure 2). Whereas the absolute numbers of transitional B cells were slightly decreased in aged subjects, the absolute numbers of mature-naive, unswitched memory, IgM only memory, switched memory and double-negative B cells, as well as plasmablasts, were similar in young and old individuals (Figure 1C). Thus, absolute numbers of total B cells and most B cell differentiation subsets remained stable with aging.

Decrease of IL-10 producing B cells in aged subjects

To determine whether aging affects cytokine production by B cells, we analysed peripheral blood B cells of young and old individuals for production of TNF-α and

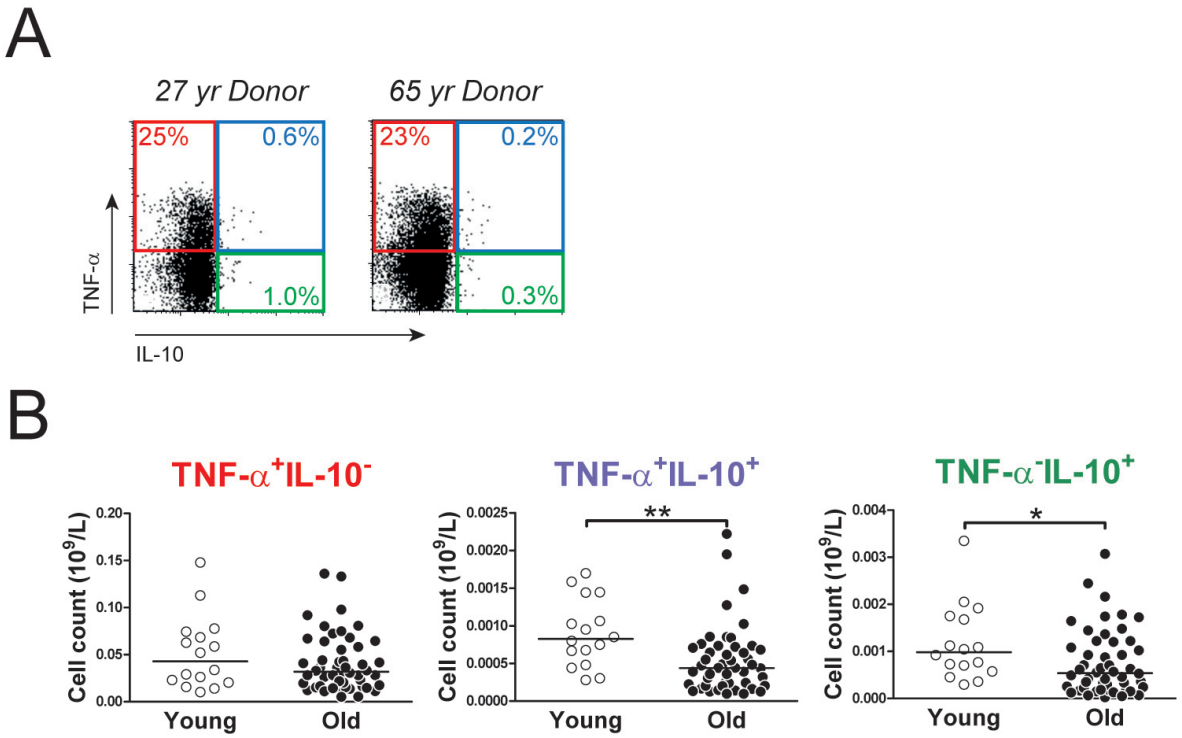

Figure 2. Effect of age on TNF-α and IL-10 production by circulating B cells. (A) Representative flow cytometric staining of TNF-α and IL-10 in $CD19^+CD22^+$ B cells of a young and an old individual. Cells were stimulated with PMA (40 nM) and calcium ionophore (2 nM) in the presence of Brefeldin A (3μM) for 4 hours. (B) Absolute numbers of $TNF\text{-}\alpha^+IL\text{-}10^-$ B cells, $TNF\text{-}\alpha^+IL\text{-}10^+$ B cells and $TNF\text{-}\alpha^-IL\text{-}10^+$ B cells in 16 young and 50 old individuals. Statistical significance by Mann Whitney U test is indicated as * $p < 0.05$ and ** $p < 0.01$.

IL-10 upon short stimulation with PMA and Ca^{2+} ionophore (Figure 2A). Absolute numbers of $TNF\text{-}\alpha^+IL\text{-}10^-$ B cells were comparable in young and old subjects (Figure 2B). In essence, IL-10 producing B cells showed an aging-associated decline, as numbers of both $TNF\text{-}\alpha^+IL\text{-}10^+$ B cells and $TNF\text{-}\alpha^-IL\text{-}10^+$ B cells were decreased in old subjects.

Next, we sought to link production of TNF-α and IL-10 by B cells to particular B cell differentiation subsets. Numbers of $TNF\text{-}\alpha^+IL\text{-}10^-$ B cells correlated positively with numbers of mature-naive, unswitched memory, IgM only memory, switched memory and double-negative B cells (Supplemental Figure 3). $TNF\text{-}\alpha^+IL\text{-}10^+$ B cells showed a positive correlation with unswitched memory, IgM only memory, switched memory and double-negative B cells. Absolute numbers of $TNF\text{-}\alpha^-IL\text{-}10^+$ B cells tended to correlate positively with switched memory B cells only. Overall, these findings implied that memory are mostly responsible for production of TNF-α and IL-10 in B cells. Additional phenotypical analysis indeed confirmed that TNF-α and IL-10 were primarily produced by the memory B cell populations (Supplemental Figure 4).

IL-10 producing B cells are maintained in aged subjects with rheumatoid factors

We next investigated the relation between declining numbers of IL-10 producing B cells in aged subjects and the prevalence of autoantibodies. Rheumatoid factors (RFs) were detected in 6% of young individuals and 12% of old individuals (Figure 3A). None of the sera from young individuals contained antinuclear

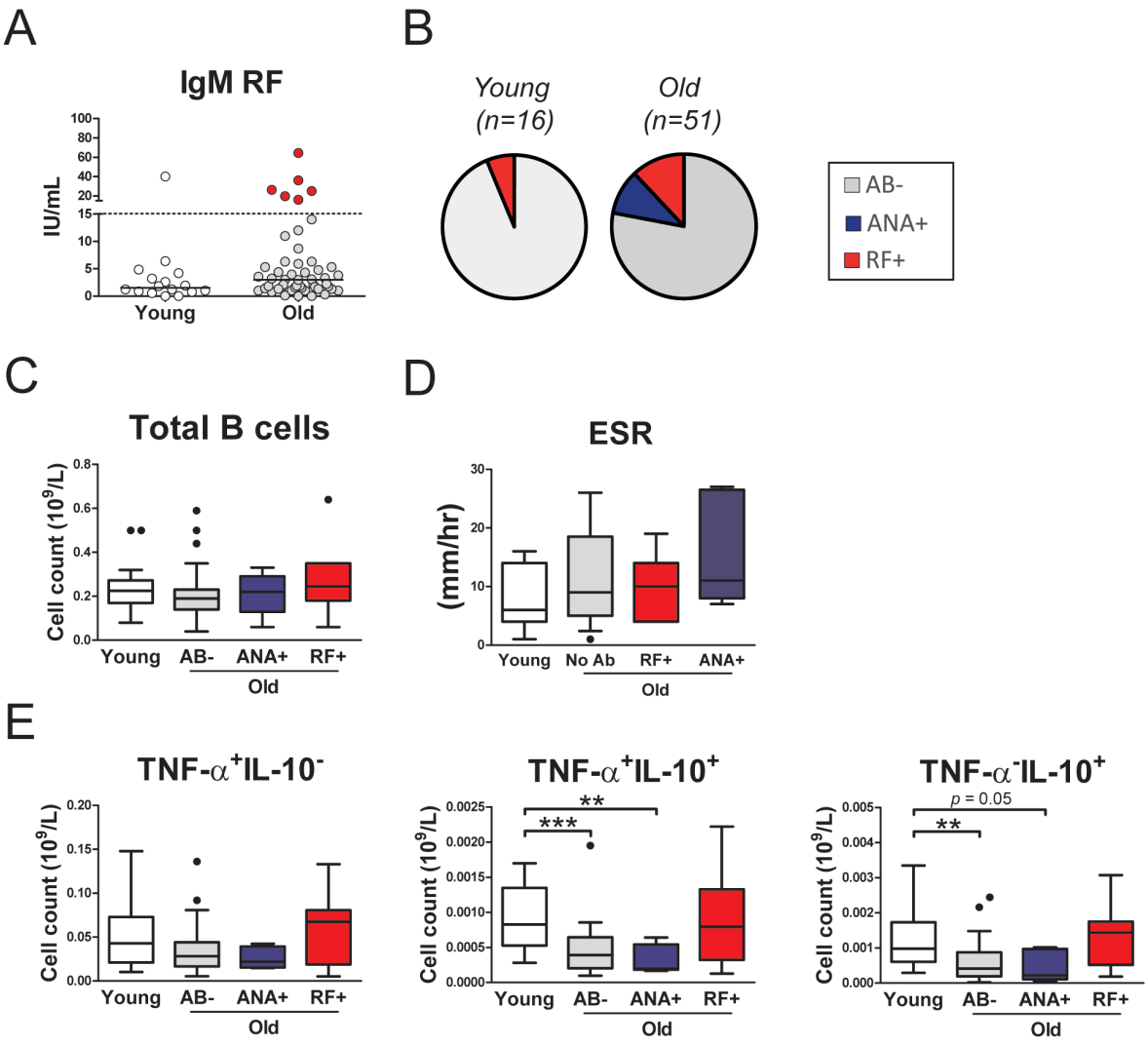

Figure 3. Relation between cytokine production in B cells and autoantibody presence. (A) Serum levels of IgM rheumatoid factors (RF) in young and old individuals. The dashed line at 15 IU/mL represents the normal value for IgM RF. (B) Pie charts showing proportions of young and old individuals that were seropositive for anti-nuclear antibodies (ANA+), seropositive for RF (RF+) or seronegative for both autoantibody types (AB-). (C) Absolute numbers of CD19+ B cells and (D) erythrocyte sedimentation rate (ESR) in 16 young individuals, as well as 50 old individuals that were ANA+ (n=5), RF+ (n=6) or AB- (n=39). (E) Absolute numbers of TNF-a^{+}IL-10^{-} B cells, TNF-a^{+}IL-10^{+} B cells and TNF-a^{-}IL-10^{+} B cells in the donors mentioned at (C and D). Statistical significance by Mann Whitney U test is indicated as ** $p < 0.01$ and *** $p < 0.001$.

antibodies (ANAs), whereas 10% of old individuals tested positive for ANAs (Figure 3B), albeit with lower titres (Supplemental Table 1). In none of the tested individuals, a combination of RFs and ANAs was observed. The absolute numbers of total $CD19^+$ B cells (Figure 3C), the erythrocyte sedimentation rates (Figure 3D) and serum levels of C-reactive protein (data not shown) were comparable in aged subjects with and without autoantibodies. Both ANA positive and autoantibody negative (AB negative) old subjects showed an aging-associated decrease of $TNFa^+IL\text{-}10^+$ and $TNFa^-IL\text{-}10^+$ B cells (Figure. 3E). In contrast, absolute numbers of these IL-10 producing B cell subsets were retained in RF positive old subjects. No aging-associated modulation of TNF-$a^+IL\text{-}10^-$ B cells was observed, irrespective of autoantibody presence.

Discussion

In this study, we show that TNF-α producing B cells are largely retained in the circulation of aged individuals, whereas numbers of IL-10 producing B cells decline. This aging-dependent decline of IL-10 producing B cells was observed in aged subjects without autoantibodies, as well as in those that had developed ANAs. In contrast, RF positive aged subjects showed a remarkable preservation of IL-10 producing B cells, when compared to other aged individuals. These findings imply that the presence of IL-10 producing B cells may differentially impact the occurrence of ANAs and RFs in the elderly.

The incidence of autoimmune diseases, such as rheumatoid arthritis, increases with age (Crowson et al, 2011). Furthermore, aging is associated with the appearance of circulating autoantibodies in healthy individuals (Nisihara et al, 2013; van Schaardenburg et al, 1993). We here confirm the increased prevalence of RFs and ANAs in aged subjects. RFs are autoantibodies directed to the constant region of the IgG heavy chain. We restricted our analysis to IgM RFs only, as IgM RFs are commonly measured in clinical practice, whereas IgA and IgG RFs are not. Earlier studies have shown that the prevalence of IgM RFs increases with age (van Schaardenburg et al, 1993). ANAs are directed towards components of the cell nucleus. The specificity of ANAs in healthy, elderly individuals remains elusive, as autoantibodies to double stranded DNA or extractable nuclear antigens are rarely observed in these individuals (Candore et al, 1997). Development of ANAs and RFs in healthy individuals may reflect polyclonal B cell activation and can occur during various types of infections (Berlin et al, 2007; Shmerling and Delbanco, 1991). Although the clinical significance of RFs and ANAs in healthy elderly individuals remains unclear, the development of RFs and ANAs implies a subtle increase in autoimmunity. Indeed, longitudinal studies indicate that the development of autoantibodies may proceed the onset of autoimmune diseases (Nielen et al, 2004).

TNF-α producing B cells were retained in the circulation of aged individuals. Accumulating evidence suggests that B cells not only contribute to autoimmunity

via secretion of autoantibodies, but also via production of pro-inflammatory cytokines (Lund and Randall, 2010). In general, TNF-α producing B cells are thought to promote low grade inflammation in the elderly (Frasca et al, 2014). Furthermore, TNF-α and IL-6 producing B cells have been directly linked to development of autoimmune diseases, such as multiple sclerosis, large vessel vasculitis and polymyalgia rheumatica (Barr et al, 2012; van der Geest et al, 2014a). Therefore, preservation of pro-inflammatory B cells may represent an important risk factor for the development of aging-associated autoimmunity.

IL-10 producing B cells were decreased in the circulation of aged individuals. These B cells may play a dual role during autoimmune responses. IL-10 producing B cells not only suppress cellular immune responses by T cells and monocytes (Blair et al, 2010; Iwata et al, 2011; Lepse et al, 2014), but may also promote the differentiation of antibody secreting cells (Heine et al, 2014). Recently, Duggal et al. have reported that transitional B cells of aged individuals show a decreased ability to differentiate into IL-10 producing B cells (Duggal et al, 2013). We here extend these in vitro findings by showing that the actual number of IL-10 producing B cells is decreased in the circulation of old subjects.

Whereas an aging-associated decrease of IL-10 producing B cells was observed in autoantibody negative and ANA positive subjects, IL-10 producing B cells were remarkably well preserved in RF positive subjects. This latter finding may seem in contrast with the prior observation that the potential of transitional B cells to differentiate into IL-10 producing B cells correlates inversely with RF levels in sera of aged subjects (Duggal et al, 2013). A direct comparison with our data, however, remains difficult, as we enumerated actual numbers of IL-10 producing B cells in the circulation of aged individuals. So far, it remains to be established why the presence of RFs and ANAs is associated with different maintenance of IL-10 producing B cells. One explanation would be that IL-10 producing B cells differentially impact the development of RFs and ANAs. For instance, IL-10 producing B cell may supress T helper cells (Blair et al, 2010), which are likely more important for the production of IgG ANAs rather than IgM RFs. Of interest, various studies imply that IL-10 potently promotes the production of RFs (Perez et al, 1995; Van Esch et al, 1997). Further studies are needed to determine if IL-10 producing B cells indeed differentially impact the development of RFs and ANAs in the elderly.

Conclusions

We demonstrate that the number of circulating, TNF-α producing B cells is maintained in the elderly, whereas IL-10 producing B cells are decreased. This decline in IL-10 producing B cells was restricted to aged subjects that were anti-nuclear antibody (ANA) positive and to those that were seronegative for both ANAs and rheumatoid factors (RFs). Interestingly, the presence of RFs was associated with a remarkable maintenance of IL-10 producing B cells in aged

subject. These findings imply a differential impact of IL-10 producing B cells on the development of ANAs and RFs in the elderly.

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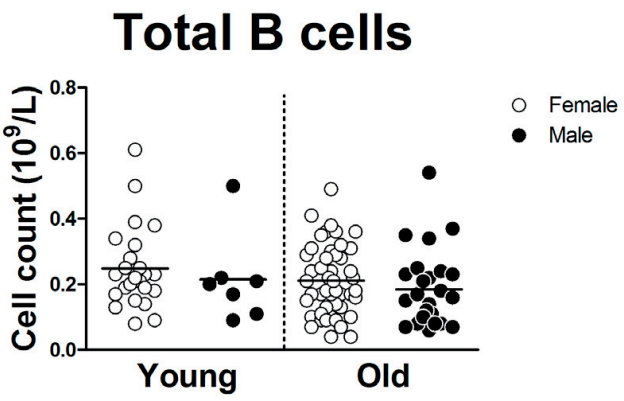
Supplementary materials

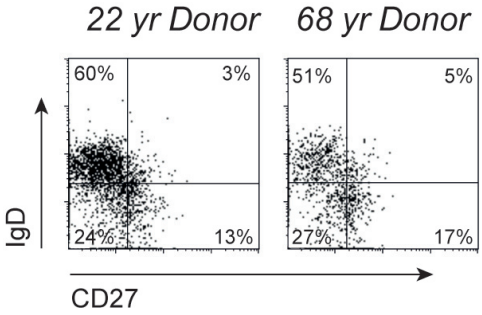
Supplemental Table 1. Anti-nuclear antibody (ANA) titre and pattern in aged individuals.

	ANA titre	ANA pattern
Aged donor A	1:40	Speckled
Aged donor B	1:40	Speckled
Aged donor C	1:40	Speckled
Aged donor D	1:80	Nucleolar
Aged donor E	1:80	Speckled

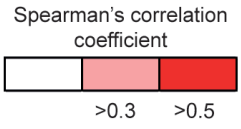
Five out of 51 aged individuals (age ≥ 60) demonstrated slightly elevated ANA titres of 1:40 or 1:80. A titre < 1:40 was considered normal.



Supplemental figure 1. Circulating B cell numbers in male and female donors. (A) Absolute numbers of CD19+ B cells in peripheral blood of 31 young (24 female, 7 male) and 73 old (47 female, 26 male) individuals.

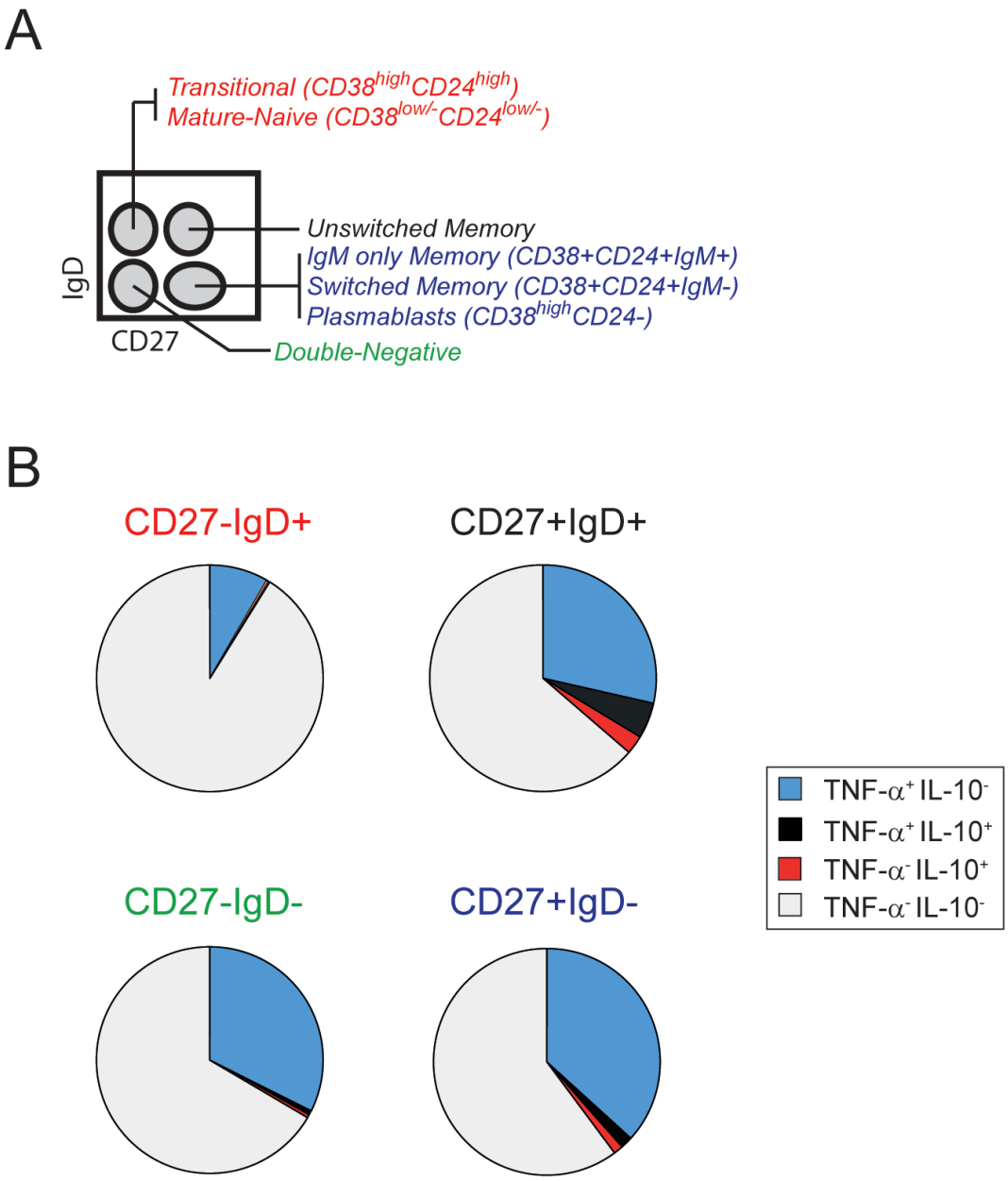


Supplemental figure 2. CD27 and IgD staining in B cells. Representative CD27/IgD staining in peripheral blood CD19+ B cells from a young and an old healthy individual.



	Transitional	Mature-Naive	Unswitched Memory	IgM only Memory	Switched Memory	Double-Negative	Plasma-blasts
TNF-α$^+$IL-10$^-$ B cells	0.16	0.39*	0.49*	0.59**	0.67**	0.61**	0.30
TNF-α$^+$IL-10$^+$ B cells	0.12	0.25	0.51**	0.66**	0.68**	0.50*	0.34
TNF-α$^-$IL-10$^+$ B cells	-0.10	0.00	0.28	0.22	0.37†	0.34	0.22

Supplemental figure 3. Spearman's correlation coefficients of the 3 TNF-α/IL-10 defined B cell populations and the 7 distinct B cell differentiation subsets. Data are representative for 26 individuals (age 19-83). Statistical significance is indicated as * $p < 0.05$ and ** $p < 0.01$. † represents a p value of 0.07.



Supplemental figure 4. Production of TNF-α and IL-10 in distinct B cell differentiation subsets. Blood samples of 7 healthy, young individuals were stimulated with PMA (50 nM) and calcium ionophore (2 nM) in the presence of Brefeldin A (3 μM) for 4 hours. **(A)** Intracellular TNF-α and IL-10 was measured in four distinct CD27/IgD defined B cell differentiation subsets. **(B)** Pie charts represent median proportions of TNF-α^+IL-10^-, TNF-α^+IL-10^+, TNF-α^-IL-10^+ and TNF-α^-IL-10^- cells among the four CD27/IgD defined B cell differentiation subsets.